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(54) Method for stabilizing extra-chromosomal elements in bacteria during cultivation, extra-chromosomal elements, transformed bacteria and a method of producing a desired product in transformed bacteria.

(57) A novel method for stabilization of extra-chromosomal elements in bacteria during cultivation without use of antibiotics in the medium has been developed. The method comprises transformation of a host bacterium having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope with an extra-chromosomal element capable of suppressing the requirement caused by the chromosomal gene defect of the host bacterium. The invention furthermore provides for such transformed bacteria and stabilized extra-chromosomal elements and for a method for producing desirable products by inserting a gene for the desirable product into a stabilized extra-chromosomal element and cultivation of suitable host bacteria containing the stabilized extra-chromosomal element comprising the gene for the desirable product. It was demonstrated that according to the principles of this method, a plasmid including the *dal* gene of *Bacillus subtilis* and a gene encoding a desirable product can be maintained in an appropriate *dal*"*B. subtilis* host in conventional media. The method thus allows for improved product yield and quality.

This invention relates to a novel method for stabilizing extra-chromosomal elements in bacteria during cultivation without the need for addition of antibiotics or other undesirable components to the growth medium with the purpose of increasing 5 yields or quality of fermentation products.

#### DESCRIPTION OF THE PRIOR ART

Microorganisms harbouring extra-chromosomal genetic elements, for example plasmids, are, generally speaking, unstable in the sense that the elements may frequently be irreversibly 10 lost. This instability is particularly pronounced if the plasmid is not indigenous to the host microorganism for instance because it comprises genes from other organisms or has been constructed by gene splicing.

To increase plasmid stability, an antibiotic or another 15 bioactive compound to which the plasmid but not the chromosome confers resistance is commonly added to the medium used for cultivation of the microorganism. In such a medium only those cells retaining the plasmid with the antibiotic resistance gene proliferate. The major disadvantages of this approach are that it 20 requires large scale growth of antibiotic resistant microorganisms, addition of expensive antibiotics to the growth medium with possible adverse effects on the environment and subsequent extensive purification to remove the antibiotic from the desired product.

25 Complementation of an auxotrophic mutation of the host chromosome is another known method for stabilization of plasmids. This approach, however, seriously restricts the composition of the growth medium and requires cultivation in a growth medium that does not contain the nutrient required by the host microorganism thereby limiting the options available for improving 30 productivity.

The object of the present invention is to provide a method for stabilization of extra-chromosomal elements in transformed bacteria, without the necessity of using antibiotics and without severe restrictions on the composition of the growth media.

It is another object of the present invention to provide stabilized extra-chromosomal elements and transformed bacteria containing such stabilized extra-chromosomal elements.

It is a further object of the present invention to provide a method for producing desirable products in transformed bacteria without the necessity of using antibiotics or special restrictions on the composition of the growth media.

Other objects of the invention will become apparent to those skilled in the art to which it pertains.

#### 15 BRIEF STATEMENT OF THE INVENTION

The invention resides in the discovery that extra-chromosomal elements can be maintained in host bacteria during cultivation in ordinary media if the extra-chromosomal elements harbour a certain function which under the given conditions is required for normal growth of the host.

When transforming a host bacterium having a mutation, a deletion or another defect in a gene encoding a function which under the given conditions is required for normal growth of the host with for instance a plasmid harbouring a gene encoding this function, only cells transformed with and maintaining the plasmid will survive because only in such cells the requirement of the host cells is supplemented by the plasmid. However, permanent plasmid maintenance is only assured if transfer of genetic information from plasmid to chromosome cannot take place and the rate of spontaneous mutation of the host bacteria into a mutant with no such requirement is insignificant.

According to a first aspect of the present invention there is provided a method for stabilizing extra-chromosomal elements in bacteria during cultivation, said method comprising:

- providing of an extra-chromosomal element containing a DNA-  
5 sequence encoding a structural or functional component being needed for the synthesis or maintenance of the cell envelope;  
and transforming a host bacterium having a defect in the chromosomal gene for such structural or functional component with the extra-chromosomal element containing said DNA-sequence;  
10 whereby the requirement caused by the chromosomal gene defect of the host bacterium is suppressed by the extra-chromosomal element and loss of the extra-chromosomal element during cultivation is insignificant.

The method furthermore includes construction of a host  
15 bacterium from which suppression of the chromosomal defect by spontaneous mutation is insignificant and construction of an extra-chromosomal element from which the DNA-sequence supplementing the chromosomal defect of the host bacterium cannot be transferred to the chromosome of the host separately from the rest of  
20 the extra-chromosomal element.

As used herein the term "extra-chromosomal elements" means plasmids, bacteriophages or any genetic material which is not normally present in the host bacteria either as independent molecules or integrated into the chromosome. Preferred extra-  
25 chromosomal elements are plasmids although use of bacteriophages and other vector systems or integration of DNA into the chromosome may illustrate the present invention to those skilled in the art.

According to a further aspect of the present invention  
30 there is provided a transformed bacterium having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope and harbouring an extra-chromosomal element capable of suppressing the requirement caused by the chromosomal defect of the host bacterium.

35 The present invention furthermore provides a method for producing desired products (i.e. DNA, RNA, peptides, and proteins) in transformed bacteria which comprises:

providing a combined extra-chromosomal element containing i) a DNA-sequence encoding a structural or functional component being needed for the synthesis or maintenance of the cell envelope and ii) a gene encoding said desired product;

5 transforming a suitable host bacterium having a defect in the chromosomal gene for said structural or functional component with said combined extra-chromosomal element;

cultivating the transformed bacterium in a suitable nutrient medium; and recovering of the desired product from the culture 10 medium.

The present invention also comprises a method for the production of a desired product in transformed bacteria which method comprises:

cultivating in a suitable nutrient medium bacteria having a 15 defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope, said bacteria containing a combined extra-chromosomal element being capable of suppressing the requirement caused by the chromosomal defect of the host bacterium, said combined extra-chromosomal element also 20 containing a DNA-sequence encoding said desired product; and recovering the desired product from the culture medium.

Suitable host bacteria are bacteria belonging to the *Bacillus* or *Enterobacteriaceae* species, e.g. *Bacillus subtilis* and *Escherichia coli* although use of other suitable bacteria may 25 be evident to those skilled in the art.

#### DETAILED EXPLANATION OF THE INVENTION

In the description the following terms are employed:

dal gene: the gene for D,L-alanine racemase.

dal<sup>+</sup> gene: a functional gene (wild type) for D,L-alanine 30 racemase.

dal-1 gene: a gene with a mutation in the D,L-alanine racemase gene normally causing requirement for external D-alanine.

dal<sup>-</sup> host: a host having a mutation in the dal gene (herein 35 normally requiring external supply of D-alanine for growth).

dal<sup>+</sup> host: a host with a wild type dal gene.

Dal<sup>+</sup> host: a host without requirement for external D-alanine.

Dal<sup>-</sup> host: a host with requirement for external D-alanine.

Cam <sup>R</sup> :	chloramphenicol resistance.
Kan <sup>R</sup> :	kanamycin resistance.
Amp <sup>R</sup> :	ampicillin resistance.
bla:	gene for $\beta$ -lactamase causing Amp <sup>R</sup> .
5 cat:	gene for chloramphenicol acetyl transferase causing Cam <sup>R</sup> .
amyM:	gene for a maltogenic amylase.

For most bacteria, the ability to grow and divide depends on a well sustained cell envelope (i.e. cell membranes, 10 cell walls and related structures). Disruption or disintegration of the envelope usually leads to lysis or cessation of growth.

One of the components indispensable for a stable cell envelope in Bacillus subtilis and most other bacteria is D-alanine. D-alanine is an essential part of the cell wall for 15 which it serves to crosslink polysaccharide chains thus conferring the cell with the necessary rigidity. D-alanine is not present in most conventional growth media and normally many bacteria including B. subtilis and E. coli synthesize this amino acid from L-alanine by means of the enzyme D,L-alanine racemase 20 and do not require an external source of D-alanine for growth. Some mutants need an external supply of D-alanine for growth as for instance dal-1 mutants of B. subtilis in which the D,L-alanine racemase gene has been damaged due to the mutation (Freese et al., Proc.Natl.Acad.Sci. 51:1164 - 72, 1964, Dul et 25 al., J. Bacteriol. 15:1212-14, 1973). Other mutants may require an external supply of other metabolites for maintenance of the cell envelope, as for instance diaminopimelic acid, D-glutamic acid, and N-acetylg glucosamin.

According to the present invention it has been proven 30 that plasmids harbouring a functional gene for a D,L-alanine racemase assure their maintenance in a dal- host if the dal+ gene cannot be transferred from plasmid to chromosome and if the frequency of spontaneous mutation of the host to a Dal+ phenotype is insignificant and if external D-alanine is not available to 35 the cell.

Thus by inserting a gene for a desirable product in an appropriate dal<sup>+</sup> plasmid and culturing the plasmid in an appropriate dal<sup>-</sup> host, the dal<sup>+</sup> plasmid will be maintained in the cell population during growth ensuring high yields of the desirable product being expressed during the autonomous replication of the plasmid. As D-alanine is absent in many conventional growth media, no further restrictions is applied on these media.

Accordingly, the present invention represents a convenient method for stabilizing extra-chromosomal elements carrying genes for desirable products during cultivation.

To ensure that the rate of spontaneous mutation of the host to a Dal<sup>+</sup> phenotype is insignificant, it may be necessary to delete a part of the dal gene.

However, by combination of such host containing a deletion of a part of the dal gene necessary for expression of the Dal<sup>+</sup> phenotype with a dal<sup>+</sup> plasmid harbouring the entire dal<sup>+</sup> gene and segments homologous to the DNA that flanks both sides of the deletion on the chromosome, transfer of the dal<sup>+</sup> allele from plasmid to chromosome may take place by homologous crossover. To avoid such homologous crossover a host was constructed in which the dal deletion extended into the segments flanking the dal gene on the chromosome. Also a plasmid was constructed which harbours a functional dal<sup>+</sup> gene but not DNA homologous to the DNA that flanks both sides of the deletion on the chromosome. Combination of this latter plasmid with forementioned dal<sup>-</sup> host constitutes the preferred host-vector pair.

Alternatively, dal<sup>+</sup> allele transfer may be prevented either by using a recombination deficient host or by using an extra-chromosomal dal<sup>+</sup> gene with no or little DNA-homology with the chromosome of the host bacterium.

The dal<sup>+</sup> gene is preferably derived from a B. subtilis strain as explained in further detail in the following. Besides the dal<sup>+</sup> gene the plasmid should also include a part for plasmid replication, e.g. the replication functions from the high-copy plasmid pUB110 for replication in Bacillae or other gram positive bacteria or the replication function of pBR322 for replication in Enterobacteriaceae or other gram-negative bacteria.

Production of a desirable product according to the present invention is illustrated by production of a maltogenic amylase from a *Bacillus* strain (NCIB 11837) expressed by its own promoter. Other examples of desirable products which may be produced according to the present invention are other kinds of amylases, amyloglycosidases, pullulanases proteinases, lipases, hormones, and other enzymes or eukaryotic proteins and peptides.

The invention will be further described with reference to the accompanying drawings in which

- 10 fig. 1 illustrates the construction of plasmids pDN691, pDN770, pDN820, and pDN1050,
- fig. 2 illustrates the construction of pDN1122,
- fig. 3 illustrates the construction of plasmids pDN1090, pDN1120, pDN1222, and pDN1277 and a map of restriction enzyme sites on pDN1000,
- 15 fig. 4 illustrates the construction of plasmids pDN1130 and pDN1290,
- fig. 5 illustrates the construction of plasmid pDN1274, and
- 20 fig. 6 illustrates the construction of plasmid pDN1800.

The method of the invention allows the cultivation of transformed bacteria in antibiotic-free media. It should be understood, however, that such is not a condition for operability of the invention, only a consequence thereof. If it becomes useful or preferable to cultivate the transformed bacteria of the invention in the presence of antibiotic(s), it can, of course be done.

#### DETAILED DESCRIPTION

The dal<sup>+</sup> gene was obtained from DN497, a derivative of 30 *Bacillus subtilis* 168 (Spizizen, Proc.Natl.Acad.Sci. 44, 1072-78, 1958). Chromosomal DNA was completely digested with appropriate restriction enzymes and ligated with a plasmid pDN691 conferring resistance to chloramphenicol and kanamycin and capable of replicating in *B. subtilis*. The ligated DNA was transformed into 35 a D-alanine requiring *B. subtilis dal-1* selecting for complementation of the D-alanine requirement. A Dal<sup>+</sup> transformant which had concomitantly become Cam<sup>R</sup> (chloramphenicol resistant) contained a recombinant plasmid derived from pDN691 on which the Dal<sup>+</sup> and Cam<sup>R</sup> phenotypes were linked. Possibly due to homologous 40 recombination between chromosome and plasmid only minute amounts of plasmid could be detected in the transformant.

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To avoid recombination and subsequent integration of plasmid into the chromosome, plasmid was prepared from this  $Dal^+$   $Cam^R$  transformant and transformed into a D-alanine requiring and recombination deficient strain DN733 dal<sup>-</sup> recE<sup>-</sup> selecting for 5  $Dal^+$ . The transformants concomitantly became  $Dal^+$   $Cam^R$  and  $Kan^R$  (kanamycin resistant).

The transformants contained small amounts of a plasmid of about 16 kb. From this plasmid, a 2.0 kb  $ClaI-SphI$  fragment conferring a  $Dal^+$  phenotype was cloned on a 2.6 kb fragment on 10 plasmid pDN820:  $Cam^R$  in strain DN608: dal<sup>-</sup> selecting for  $Cam^R$   $Dal^+$  to give a recombinant plasmid pDN1000 of 4.6 kb. This plasmid could however replace the dal-1 mutation on the chromosome of recombination proficient strains (e.g. DN608) with the 15 dal<sup>+</sup> allele by homologous recombination upon which the selection of a  $Dal^+$  phenotype without concomitant  $Cam^R$  selection would not anymore assure maintenance of the plasmid.

To prevent transfer of the dal<sup>+</sup> allele to the chromosome by homologous recombination and subsequent loss of plasmid and to reduce the frequency of mutations causing a  $Dal^+$  phenotype, a deletion of both the dal gene in the host and a neighbouring segment was made in the host chromosome: the cloned dal gene was cut with restriction enzymes EcoR1 and EcoR5 and subsequently digested with exonuclease Bal31. The digestion mixture was ligated and transformed into DN608 dal<sup>-</sup> selecting for 25  $Cam^R$ . Transformants containing plasmids with deletions in the dal gene were identified as  $Dal^-Cam^R$ . To construct a host strain with the appropriate deletion in the dal gene, a dal<sup>+</sup> host was transformed with one of the deletion plasmids, pDN1274  $Cam^R$  dal<sup>-</sup>. Selecting for  $Cam^R$ , about 0.1% of the transformants were  $Dal^-$  30 presumably due to replacement of the chromosomal dal<sup>+</sup> gene with the dal<sup>-</sup> deletion from pDN1274 by homologous recombination. Following spontaneous loss of pDN1274 upon growth in the presence of D-alanine and absence of chloramphenicol, the plasmid free strain DN1280 remained  $Dal^-$ . Southern blotting analysis of 35 chromosomal DNA from DN1280 showed that it indeed harboured the expected dal deletion. This dal<sup>-</sup> deletion host, strain DN1280, was transformed with a dal<sup>+</sup> plasmid (e.g. pDN1090) harbouring both the entire dal<sup>+</sup> gene and segments homologous to the DNA

flanking both sides of the deletion on the chromosome. Transfer of the dal<sup>+</sup> gene from plasmid to chromosome by homologous recombination could therefore take place and the host could be converted into dal<sup>+</sup>. Hence, maintenance of the plasmid was no longer a condition for a Dal<sup>+</sup> phenotype and the plasmid was frequently lost. A dal<sup>+</sup> plasmid, pDN1277, was then constructed harbouring the entire functional dal<sup>+</sup> gene, but not DNA homologous to the DNA that flanks the deletion on the host chromosome as explained above. Therefore, transfer of dal<sup>+</sup> from plasmid to chromosome by homologous recombination could not take place upon transformation of the dal<sup>-</sup> deletion host with pDN1277. Hence DN1280 is a suitable dal<sup>-</sup> host in D-alanine free media for dal<sup>+</sup> plasmids which do not harbour the DNA including the EcoR5 site which normally flanks the dal gene on the chromosome but which has been deleted in DN1280.

In order to ascertain that cloned genes of scientific or commercial interest indeed could be maintained on plasmids in D-alanine free media by the above host-vector system, the gene for the maltogenic amylase from Bacillus C599 (NCIB 11837) was transferred to a dal<sup>+</sup> plasmid. Two plasmids pDN1130 and pDN1290 were constructed. Both plasmids harbour the replication functions of plasmid pUB110 and functional dal and amyM genes. Neither plasmids confer resistance to any antibiotic. Plasmids pDN1130 and pDN1290 were transformed into the above B. subtilis strain DN1280. Strain DN1280 harbours as mentioned above a chromosomal deletion which includes both a part of the dal gene necessary for expression of the Dal<sup>+</sup> phenotype and an adjoining segment which is not required for the Dal<sup>+</sup> phenotype it be part of the dal gene senso strictu or not. This latter part of the deletion is not harboured by pDN1290. Accordingly, the dal<sup>+</sup> gene cannot be transferred from pDN1290 to the chromosome by double homologous recombination. Plasmid pDN1130, on the other hand, harbours both the entire segment which is deleted on the chromosome and adjoining segments. For this reason transfer of the dal<sup>+</sup> gene from plasmid to chromosome and subsequent plasmid loss may take place.

The obtained transformed strains DN1297 (=DN1280 pDN1130) and DN1300 (=DN1280 pDN1290) were tested for plasmid stability during cultivation (see example 7). When culturing DN1297 homologous crossover restoring the dal<sup>+</sup> gene on the 5 chromosome took place and both the plasmid and the Amy<sup>+</sup> phenotype were frequently lost.

When culturing DN1300 no loss of the Amy<sup>+</sup> phenotype conferred by the plasmid was observed upon growth in media without D-alanine. If D-alanine was added to the medium, however, 10 cells frequently lost the plasmid and became Amy<sup>-</sup> Dal<sup>-</sup>. Thus, stable maintenance of a plasmid harbouring a non-indigeneous gene of commercial interest, in an antibiotic-free medium was demonstrated.

To demonstrate that plasmids may be maintained also in 15 gram-negative organisms by suppression of a D-alanine requirement the dal gene of B. subtilis was cloned on a plasmid in a D-alanine requiring Escherichia coli mutant and shown to complement the requirement.

Further details of the present invention will appear 20 from the following examples.

#### EXPERIMENTAL PART

Preparation of plasmids and chromosomal DNA and transformation of Bacillus subtilis and E. coli were conducted according to the following general procedures. Digestion with restriction enzymes, Bal 31 nuclease treatment, oligo-DNA-linker insertion and ligation with T4-ligase of DNA were performed with enzymes from New England Biolabs under the conditions suggested by the supplier.

##### Strains

30 All Bacillus subtilis strains were derivatives of Bacillus subtilis 168 (Spizizen, Proc.Natl.Acad.Sci., 44: 1072-78, 1958). RUB200: aroI906, amyE07, amyR2 was obtained from Dr. Frank Young, University of Rochester, New York. SL438:trpC2 (sporulation and protease deficient) was obtained from Dr. Kim

Hardy, Biogen, Geneve. DN497: amyE07, amyR2 is an aro<sup>+</sup> transformant of RUB200 with chromosomal DNA from SL438, QB1133: aroI906, metB5, sacA321, amyE was from Dr. Georges Rapoport, IRBM, Paris. QB1130: dal, metB5, sacA331, amyE was obtained from 5 the Bacillus Genetic Stock Center, Columbus, Ohio. DN608: dal-1, metB, sacA, amyE was an aro<sup>+</sup>, dal-1 transformant of QB1133 with chromosomal DNA from QB1130. MT120: leuB6, recE4  $r_m^- m_m^-$  was obtained from Dr. Teruo Tanaka, Mitsubishi-Kasei Institute of Life Sciences, Tokyo. DN773: dal-1, amyE, recE, sacA was a met<sup>+</sup> 10 recE transformant of DN608 with chromosomal DNA from MT120. DN606 is DN608 transformed with plasmid pUB110.

Escherichia coli strain TKL10: thr-1 leuB6 codAl trp-64 pyrF101 his-108 thyA6 argG66 ilvA634 thi-1 alr-1 deoC1 lacY1 tonA21 tsx95 supE44 (Wijsman, Genet. Res., Camb. 20: 269-77, 15 1972) was obtained from Dr. Barbara Bachmann the E. coli Genetic Stock Center Connecticut, U.S.A. (CGSC 5466).

#### Plasmids

pUC9 of 2.7 kb confers resistance to ampicillin and was derived from pBR322 (Vieira et al., Gene 19: 259 - 68, 1982). 20 pBR322 of 4.4 kb confers resistance to ampicillin and tetracycline (Bolivar et al., Gene 2: 95 - 113, 1977).

Plasmids pUB110 and pBD64 (Gryczan et al., J. Bacteriol. 134: 318 - 329, 1978, and Gryczan et al., J. Bacteriol. 141: 246 - 53, 1980) were isolated from B. subtilis 25 strains BD366 and BD624, respectively. pUB110 and pBD64 both confer resistance to kanamycin and pBD64 also to chloramphenicol. B. subtilis strains BD366 and BD624 can be obtained from the Bacillus Genetic Stock Center, Columbus, Ohio, USA (strain file number BGSC 1E6 and 1E22). Plasmid pDN452 of 7.6 kb confers 30 resistance to chloramphenicol and kanamycin and harbours the structural gene for a maltogenic amylase from Bacillus subtilis NCIB 11837. The construction of pDN452 is described in EP patent application No. 84301994.4.

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I. Transformation of *B. subtilis*

Competent Bacillus subtilis cells were prepared according to Yasbin et al. (J. Bacteriol. 121: 296 - 304, 1975). Cells were then harvested by centrifugation (7000 rpm, 3 min.), 5 resuspended in one tenth volume of supernatant including 20% glycerol, frozen in liquid nitrogen and stored at -70°C. For transformation, frozen cells were thawed at 42°C and mixed with one volume buffer (Spizizen's minimal medium (Spizizen, Proc. Natl. Acad. Sci. USA 44:1072 - 78, 1958)) with 0.4% glucose, 0.04 M MgCl<sub>2</sub>, and 0.002 M EGTA). DNA was added and the mixture incubated with shaking at 37°C for 20 min. Cells were then plated on appropriate selective media.

II. Transformation of *E. coli*

An overnight culture of E. coli K-12 strain No. 802 in LB 15 (10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl per liter water, pH 7.0) was diluted 100 fold in 500 ml LB and grown at 37°C to OD<sub>450</sub> = 0.4. The culture was chilled, left 15 min. on ice, spun for 15 min. at 3000 rpm (in a Sorvall GS3 rotor), resuspended in 200 ml cold 0.1 M CaCl<sub>2</sub>, 20 left on ice for 20 min., spun for 10 min. at 3000 rpm, resuspended in 5 ml cold 0.1 M CaCl<sub>2</sub> and left on ice for 20 hours. Cold glycerol was then added to 10% and aliquotes were frozen in liquid nitrogen and stored at -70°C. Frozen cells were thawed on ice, DNA was added, the mixture 25 incubated 45 min. on ice, 2 min. at 37°C and then plated on an appropriate selective medium.

III. Preparation of plasmids from *E. coli*

E. coli was grown over night in 250 ml LB, 0.4% glucose and an appropriate antibiotic. Cells were harvested by centrifugation and resuspended in 4 ml Buffer 1 (0.025 M Tris·HCl, pH = 8.0, 0.01 M EDTA, 0.05 M glucose, 2 mg/ml lysozyme). The suspension was incubated at 0°C for 15 min. and then mixed with 8 ml Buffer 2 (0.2 M NaOH, 1% SDS). Then 6 ml

Buffer 3 (3M NaAcetate, pH = 4.8) was added, the mixture kept at 0°C for 60 min. followed by centrifugation for 20 min. at 19000 rpm (ca. 45000 g in Sorvall SS34 rotor). The supernatant was precipitated with 0.6 vol cold isopropanol 5 and resuspended in 1.2 ml 5TE (0.05 M Tris·HCl, pH = 8.0, 0.005 M EDTA), plus 20 µl boiled RNase A (Boehringer) (2 mg/ml). 30 min. later the solution was layered on top of 4.0 ml Buffer 4 (80 g CsCl plus 56 ml 5TE) and 0.1 ml EtBr (10 mg/ml ethidium bromide) in a VTi65 tube. The mixture was 10 centrifuged at 45000 rpm for 20 h. The plasmid was then removed from the tube, dialyzed and extracted as described in section VI.

#### IV. Preparation of plasmids from *B. subtilis*

Plasmid was prepared as described for *E. coli* strains (see 15 section III) but with the following modifications. Growth was in LB including 0.01 M potassium phosphate, pH = 7.0 and an appropriate antibiotic (e.g. 6 µg/ml chloramphenicol) and if required 100 µg/ml D-alanine. After harvest, cells were incubated at 37°C with lysozyme. Buffer 2 was replaced by a 20 mixture of one volume Buffer 2 and three volumes Buffer 5 (0.2 M glycine, 0.2 M NaCl and 1% SDS). The following steps were the same as in III.

#### V. Small scale preparation of plasmids from *B. subtilis*.

Plasmid from 5 ml *B. subtilis* in LB (including 0.01 M phosphate pH = 7.0 and appropriate antibiotics and D-alanine if required) was prepared as in section IV except: 1: volumes of buffers were reduced four fold. 2: 0.5 ml phenol and 0.5 ml chloroform are added after Buffer 3. 3: After centrifugation at 19000 rpm, the supernatant was precipitated with 30 ethanol, resuspended in 400 µl Buffer 6 (0.05 M Tris·HCl pH = 8.0, 0.1 M NaAcetate), the plasmid was again precipitated,

resuspended in 400  $\mu$ l Buffer 6, precipitated, washed and resuspended in 100  $\mu$ l TE (0.01 M Tris-HCl, pH = 8.0, 0.001 M EDTA) with 1  $\mu$ g/ml boiled RNase A (Boehringer).

VI. Preparation of chromosomal DNA from *B. subtilis*.

5 A pellet of frozen cells from about 50 ml culture was resuspended in 1.1 ml Buffer (0.05 M Tris-HCl, pH = 7.4, 0.1 M NaCl, 25% sucrose). 100  $\mu$ l lyzosyme (25 mg/ml) and 150  $\mu$ l EDTA (0.5 M, pH = 8.0) were added. The mixture was incubated at 37°C for 30 min. 2 ml 0.2% SDS was added followed by 10 incubation for 30 min. at 37°C. 1 g CsCl and 0.05 ml EtBr (10 mg/ml) were added per 0.95 ml mixture and the mixture was centrifuged at 45000 rpm, 15°C, for 20 hours in a VTi65 rotor (Beckman).

The DNA was located under a long wave UV lamp and removed by 15 puncturing the tube with a syringe. EtBr was extracted with isopropanol and the solution dialyzed for 2 hours against TEE (0.01 M Tris-HCl, pH = 8.0, 0.01 M EDTA). The solution was then adjusted to 8 ml with TEE and extracted twice with phenol and once with chloroform. The DNA was precipitated 20 with 0.1 M NaCl and cold ethanol and dissolved in 1 ml TE (0.01 M Tris-HCl, pH = 8.0, 0.001 M EDTA). The solution of chromosomal DNA was kept at 4°C.

Example 1

Construction of plasmid pDN1050 (Fig. 1)

25 Plasmid pBD64 was cut with restriction enzymes EcoR1 and Sph1 and a 3.6 kb fragment was ligated with a 0.56 kb EcoR1-Sph1 fragment from E. coli plasmid pBR322. The resulting plasmid pDN691 of 4.2 kb confers chloramphenicol and kanamycin resistance. A 0.4 kb Hind3-BamH1 fragment of pDN691 was replaced

with a 0.02 kb Hind3-BamH1 fragment of E. coli plasmid pUB9. The resulting plasmid pDN720 of 3.8 kb confers chloramphenicol and kanamycin resistance.

A 2.8 kb Ncol-Ncol fragment of pDN720 was replaced with  
5 a 1.8 kb Ncol-Ncol fragment of pDN770. pDN770 of 3.6 kb was a spontaneous deletion of pBD64 and confers chloramphenicol resistance. The resulting plasmid pDN820 of 2.8 kb confers chloramphenicol resistance. pDN820 was opened at the single HgiAl site and digested with Bal31 for 30 sec. at 30°C whereby a  
10 fragment of 0.1 kb was removed. The obtained linear fragments were ligated with of a Bgl2 oligonucleotide linker from New England Nuclear (No. 1001). Plasmid pDN1050 of 2.7 kb including one Bgl2 linker and conferring chloramphenicol resistance was isolated from this ligation mixture.

15 Example 2

Construction of plasmid pDN1122 (Fig. 2)

Plasmid pDN452 of 7.6 kb conferring resistance to chloramphenicol and kanamycin and harbouring the structural gene amyM for a maltogenic amylase of Bacillus C599 was digested with  
20 Aval, treated with exonuclease Bal31, ligated with EcoR1 oligonucleotide linker (Biolabs No. 1004), digested with EcoR1, ligated with T4-ligase and transformed into B. subtilis DN497:amyE selecting for Cam<sup>R</sup>. One Amy<sup>+</sup> transformant contained plasmid p520-20 of 6.6 kb. The amylase yield of p520-20 was not less than  
25 the yield of pDN452.

A 2.7 kb Ncol - Ncol fragment of p520-20 was then replaced with a 1.7 kb Ncol - Ncol fragment of pDN770 (prepared as in Example 1). The obtained plasmid pDN808 of 5.6 kb harbours amyM and confers resistance to chloramphenicol.

30 A 2.6 kb EcoR1-Sph1 fragment of pDN808 was replaced with a 2.4 EcoR1-Sph1 fragment of pDN1050 (prepared in Example 1). The resulting plasmid pDN1122 of 5.4 kb harbours amyM and confers resistance to chloramphenicol.

Example 3Cloning of the dal gene

About 3 µg chromosomal DNA from B. subtilis strain DN497 and 1 µg plasmid pDN691 Cam<sup>R</sup> Kan<sup>R</sup> were completely digested 5 with restriction enzymes BamH1 and Sph1. Chromosomal and plasmid DNA were mixed and ligated with T4-ligase and transformed into strain DN606:dal<sup>-</sup> pUB110:Kan<sup>R</sup>. Among about 200 Dal<sup>+</sup> transformants, one had concomitantly become Cam<sup>R</sup> as a suggestion that the Dal<sup>+</sup> phenotype was linked to a recombinant plasmid derived 10 from pDN691. Plasmids were prepared from this Dal<sup>+</sup> Cam<sup>R</sup> transformant and transformed into strain DN773 dal<sup>-</sup> recE<sup>-</sup> selecting for Dal<sup>+</sup>. Dal<sup>+</sup> transformants concomitantly became Cam<sup>R</sup> and Kan<sup>R</sup>. The transformants contained only very small amounts of a plasmid of about 16 kb.

15 From this plasmid a 2.0 kb Clal-Sph1 fragment conferring a Dal<sup>+</sup> phenotype was cloned in the Clal and Sph1 sites of plasmid pDN820 in strain DN608:dal<sup>-</sup> to give a recombinant plasmid, pDN1000 of 4.6 kb.

A map of restriction enzyme sites on pDN1000 is given 20 in fig. 3. The transformed strain DN1000=DN608 pDN1000 was deposited with the National Collection of Industrial Bacteria, (NCIB), Torry Research Station, Aberdeen, Scotland, on 7th December, 1984 and accorded the reference number NCIB 12029. NCIB being an international depository authorized under the Budapest 25 Treaty of 1977, affords permanence of the deposit and accessibility thereto by the public in accordance with Rules 9 and 11, respectively, of the above treaty.

The following observations ascertained that the cloned chromosomal fragment indeed included the dal gene (as defined by 30 the dal-1 mutation) and not another gene being able to suppress the D-alanine requirement:

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1: Linearized (non-replicative) plasmid transformed a dal<sup>-</sup> recipient to Dal<sup>+</sup> but not to Cam<sup>R</sup> as an indication of homologous recombination between the dal gene of the chromosome and the cloned chromosomal fragment.

5 2: About 0.2% of plasmids prepared from a host bacterium being dal-1 on the chromosome were dal<sup>-</sup> (yet with a restriction enzyme pattern indistinguishable from the original dal<sup>+</sup> plasmid). These dal<sup>-</sup> plasmids could not complement the chromosomal dal-1 mutation as an indication that the chromosomal mutation had been 10 transferred to the dal<sup>-</sup> plasmids by homologous recombination.

3: By Southern blotting analysis of chromosomal B. subtilis DNA, it was demonstrated that a chromosomal Clal-SphI fragment hybridized with a fragment of the same size from pDN1000. Thus, no major rearrangement of the dal gene had 15 occurred prior to its cloning on pDN1000.

#### Example 4

##### Construction of plasmid pDN1130 (Figs. 3 and 4)

pDN1000 (from Example 3) harbouring the dal<sup>+</sup> gene was opened at the Clal site, exonuclease digested with Bal31 and 20 ligated with a BamH1 oligonucleotide linker (No. 1017 from Biolabs). The resulting plasmid pDN1090 harbours the dal<sup>+</sup> gene and confers resistance to chloramphenicol.

pDN1122 (from Example 2) was cut with SphI and BglI and a 4.4 kb fragment was ligated with a 2.0 kb SphI-BamH1 25 fragment of pDN1090.

The resulting plasmid pDN1130 harbours the amyM<sup>+</sup> and the dal<sup>+</sup> gene but does not confer resistance to chloramphenicol.

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Example 5Construction of plasmid pDN1290 (Figs. 3 and 4)

A plasmid pDN1110 dal<sup>+</sup> Cam<sup>R</sup> of 4.5 kb was constructed by exonuclease Bal31 digestion of pDN1000 digested with SphI and 5 insertion of both a BglII and a SacI oligonucleotide linker (No. 1001 and No. 1005 from Biolabs, respectively).

pDN1110 was opened at the single EcoR5 site, exonuclease digested with Bal31 and ligated with two BglII oligonucleotide linkers (No. 1001 from Biolabs). The fusion of linker with one 10 plasmid end created a BclI site. The resulting plasmid pDN1222 harbours the dal<sup>+</sup> gene and confers resistance to chloramphenicol.

A 0.7 kb BclI-BclI fragment of pDN1222 was ligated with a 3.5 kb BamHI-BclI fragment of pDN1090 (from example 4). The 15 resulting plasmid pDN1277 of 4.2 kb harbours the dal<sup>+</sup> gene and confers resistance to chloramphenicol. Plasmid pDN1277 was transformed into strain DN1280 (see example 6) and the resulting strain DN1517 (=DN1280 pDN1277) was deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, on 7th December 1984 and accorded the 20 reference number NCIB 12030.

pDN1090 was furthermore converted into plasmid pDN1120 dal<sup>+</sup> Cam<sup>R</sup> of 4.5 kb by replacement of a 2.6 kb SphI-BamH1 fragment with a 2.5 kb SphI-BamH1 fragment from pDN1050 (prepared as described in example 1).

25 pDN1265 amyM<sup>+</sup> Cam<sup>R</sup> was constructed by Bal31 exonuclease digestion of pDN1120 digested with EcoR5 and subsequent digestion with SphI. A fragment of 2.9 kb was then ligated with SacI oligonucleotide linker (Biolabs No. 1005) and a 2.9 kb fragment of pDN1130 obtained by digestion with EcoRI and Bal31 exonuclease 30 treatment, and subsequent digestion with SphI.

The resulting plasmid pDN1265 of 5.8 kb harbours the amyM<sup>+</sup> gene and confers resistance to chloramphenicol.

pDN1265 was then cut with SphI-BglII and a 4.7 kb fragment was ligated with a 1.6 kb SphI-BglII fragment of pDN1277.

35 The resulting plasmid pDN1290 of about 6.3 kb harbours the amyM<sup>+</sup> and the dal<sup>+</sup> gene but no antibiotic resistance marker.

Example 6Construction of a host with deletion in the dal gene (Fig. 5)

By cutting 5 µg of pDN1090 in the cloned dal<sup>+</sup> gene with restriction enzymes EcoR1 and EcoR5 and subsequently digesting 5 the fragments with exonuclease Bal31 for 60 sec. ligating and transforming into DN608:dal-1, deletions were obtained which destroyed the Dal<sup>+</sup> phenotype of the plasmid. Transformation of strain DN497 with one of these deletion plasmids pDN1274 Cam<sup>R</sup> dal<sup>-</sup>, and selecting for Cam<sup>R</sup> gave about 0.1% of the transformants 10 which were Dal<sup>-</sup> presumably due to replacement of the chromosomal dal<sup>+</sup> gene with the dal<sup>-</sup> deletion from pDN1274. After spontaneously having lost pDN1274, the transformant became Cam<sup>R</sup> but remained Dal<sup>-</sup>. Southern blotting analysis of chromosomal DNA of this strain, DN1280, showed that it indeed harboured the expected dal 15 deletion. The frequency of mutations which might cause reversion of this dal deletion mutant to a Dal<sup>+</sup> phenotype was less than 10<sup>-8</sup>. DN1280 harbouring plasmid pDN1277 = DN1517 (see example 5) was deposited.

Example 720 Stability of dal<sup>+</sup> amyM<sup>+</sup> plasmids in a dal<sup>-</sup> host

To demonstrate both the stability of the host - plasmid combination DN1300 (=DN1280 pDN1290) and the importance of the absence of overlapping chromosomal and plasmid DNA segments flanking the chromosomal deletion, the following experiment was 25 undertaken:

Single colonies of strains DN1297 (=DN1280 pDN1130) and DN1300 (=DN1280 pDN1290) were resuspended in L-broth supplemented with 10 mM potassium phosphate buffer pH = 7.0 and 0.2% glucose. Half of each resuspension was inoculated into the above medium, 30 the other half into an identical medium supplemented with 200 µg/ml D-alanine. By diluting the cultures either 100 or 1000

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fold, the strains were grown for a number of generations at 37°C with good aeration either in the presence or absence of D-alanine in the medium. For each dilution, not less than  $10^7$  cells were transferred. With intervals of 1 day, the frequency of Amy<sup>+</sup> colonies (a total of about 100) were tested on L-broth plates with or without D-alanine according to the growth conditions. The results are shown in Table 1. 10 randomly chosen Amy<sup>-</sup> colonies were shown not to contain any plasmid.

Table 1

10 Frequency of Amy<sup>-</sup> cells after growth in LB medium with or without D-alanine.

Strains	Plasmid	Generations		
		51	68	85
		% Amy <sup>-</sup>		
15				
DN1297	pDN1130	2%	20%	-
DN1297 + D-ala <sup>1</sup>	pDN1130	60%	-	-
DN1300	pDN1290	0%	0%	0%
DN1300 + D-ala <sup>1</sup>	pDN1290	60%	90%	-

20 <sup>1</sup> First 20 generations in the absence of D-alanine.

The results in Table 1 demonstrate that a plasmid pDN1290 is highly unstable during unselective conditions (addition of D-alanine to the growth medium) but become stable during growth in a selective medium (without addition of a D-alanine). It is furthermore demonstrated that homologous double crossover restoring the dal<sup>+</sup> gene on the host chromosome and subsequent plasmid loss did not take place in DN1300 (no Amy<sup>-</sup> cells after growth for 85 generations) whereas the frequency of Amy<sup>-</sup> cells was 20% after 68 generations of DN1297 grown in a D-alanine free medium presumably due to transfer of the dal<sup>+</sup> gene from plasmid to chromosome and subsequent plasmid loss.

Construction of plasmid pDN1800 (fig. 6)

pDN1284 was constructed by combining the 1.4 kb Bgl2-Bgl2 fragment of pDN1222 (see fig. 3) with the 1.5 kb BamH1-Bgl2 5 fragment of pDN1050 (see fig. 3).

From pDN1284, the dal<sup>+</sup> gene was transferred on a 1.4 kb Bgl2-Sall fragment to the classical E. coli plasmid vector pBR322 which had been cut with BamH1 and Sall. The recombinant plasmid, pDN1800:Amp<sup>R</sup> (ampicillin resistant) was transformed into E. coli 10 strain TKL10 selecting for Amp<sup>R</sup> to obtain strain DN1800: pDN1800 Strain DN 1800 was deposited with the NCIB on 22th November, 1985 and accorded the reference number NCIB 12481 Accordingly permanence of the deposit and accessibility thereto by the public is ensured (vide supra). TKL10 displays a D-alanine requiring 15 phenotype at 42°C but in DN1800 this requirement is suppressed. Hence, pDN1800 harbouring the dal gene of B. subtilis is able to complement a D-alanine requirement in a E. coli strain defective in alanine racemase activity (Wild et al., Molec.Gen.Genet. 198:315-22, 1985).

20 The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

## CLAIMS

1. A method for stabilizing extra-chromosomal elements in bacteria during cultivation, said method comprising:  
providing an extra-chromosomal element containing a  
5 DNA-sequence encoding a structural or functional component being needed for the synthesis or maintenance of the cell envelope, and transforming a host bacterium having a defect in the chromosomal gene for said structural or functional component with the extra-chromosomal element containing said DNA-sequence,  
10 whereby the requirement caused by the chromosomal gene defect of the host bacterium is suppressed by the extra-chromosomal element and loss or modification of the extra-chromosomal element during cultivation is insignificant.

2. A method according to claim 1, wherein the  
15 bacterium is a Bacillus species.

3. A method according to claim 2, wherein the Bacillus species is a Bacillus subtilis.

4. A method according to claim 1, wherein the bacterium is an Enterobacteriaceae species.

20 5. A method according to claim 4, wherein the Enterobacteriaceae species is an Escherichia coli.

6. A method according to one of the preceding claims, wherein the structural or functional component causes the formation of D-alanine or suppresses a D-alanine requirement.

7. A method according to claim 6, wherein the structural or functional component is D,L-alanine racemase.

8. A method according to claim 1, wherein the host bacterium harbours a mutation in dal, the gene for D,L-alanine 5 racemase, or a deletion comprising at least part of the dal gene.

9. A method according to claim 8, wherein the mutation is a deletion of both a part of the dal gene necessary for expression of the Dal<sup>+</sup> phenotype and of a part of directly flanking DNA unnecessary for expression of the Dal<sup>+</sup> phenotype 10 whether it be part of the dal gene or not.

10. A method according to claim 1, wherein the extra chromosomal element is a plasmid or bacteriophage harbouring a functional part of the dal gene from B. subtilis.

11. An extra-chromosomal element comprising a DNA- 15 sequence encoding a structural or functional component causing the formation of D-alanine.

12. A transformed bacterium derived from a host bacterium having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope, said transformed 20 bacterium containing an extra-chromosomal element capable of suppressing the requirement caused by the chromosomal defect of the host bacterium.

13. A transformed bacterium according to claim 12, wherein the bacterium harbours a mutation in dal, the gene for 25 D,L-alanine racemase, or a deletion comprising at least part of the dal gene and the extra chromosomal element comprises a DNA-sequence encoding a structural or functional component causing the formation of D-alanine.

14. A transformed bacterium according to claim 13, 30 wherein transfer of the dal<sup>+</sup> allele from plasmid to chromosome is prevented by insufficient DNA homology.

15. A transformed bacterium according to claim 13,  
wherein transfer of the dal<sup>+</sup> allele from plasmid to chromosome is  
prevented by a recombination deficiency.

16. A transformed bacterium according to claim 12,  
5 wherein the defect in the chromosomal gene is a deletion of both  
a part of the dal gene necessary for expression of the Dal<sup>+</sup>  
phenotype and of a part of directly flanking DNA unnecessary for  
expression of the Dal<sup>+</sup> phenotype whether it be part of the dal  
gene or not.

10 17. A transformed bacterium according to claim 16,  
wherein the extra-chromosomal element harbours a part of the dal  
gene which is sufficient for expression of the Dal<sup>+</sup> phenotype but  
does not harbour segments homologous to the DNA that flanks at  
least one side of the deletion on the chromosome.

15 18. A method for the production of a desired product  
in transformed bacteria which comprises:

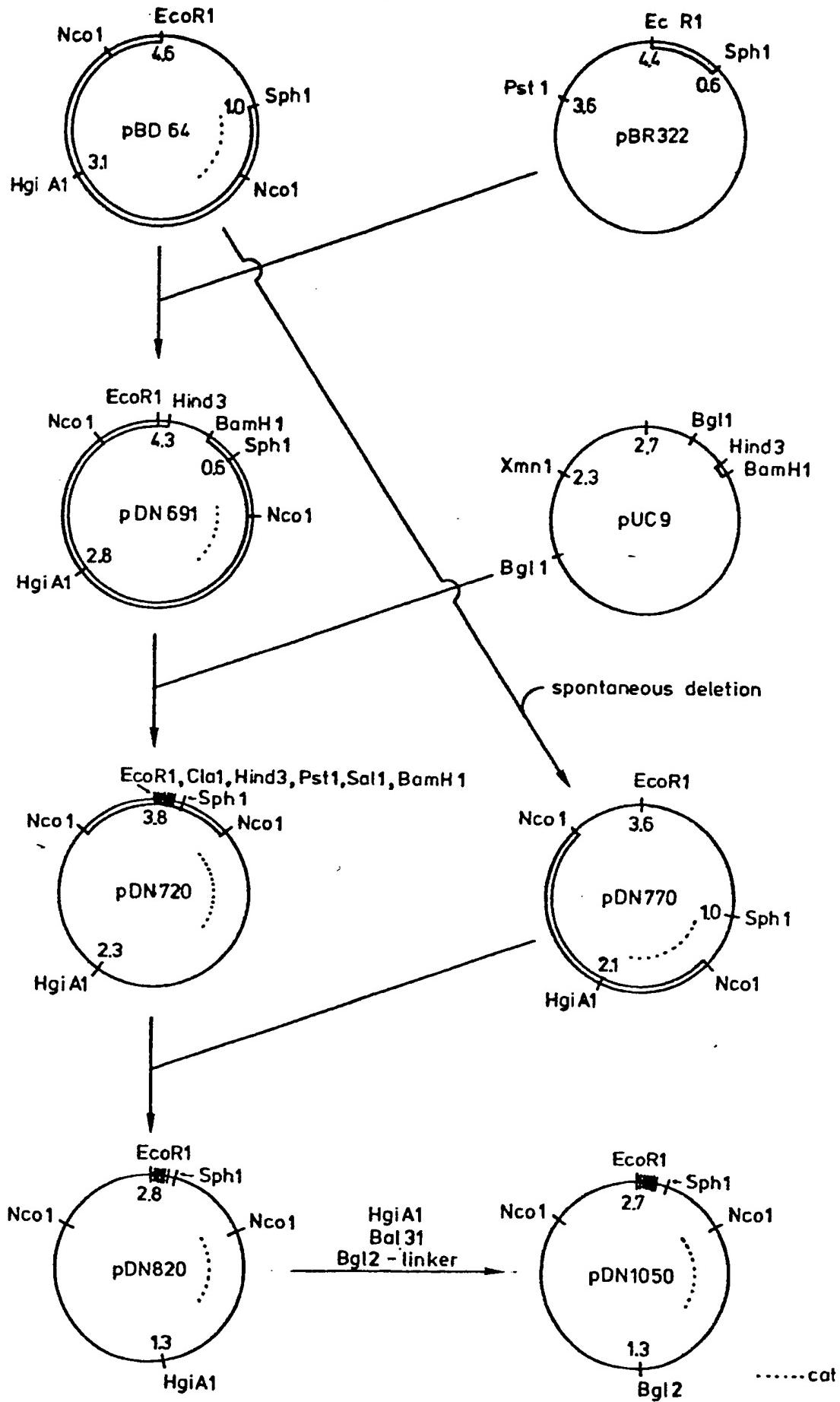
providing a combined extra-chromosomal element containing i)  
a DNA-sequence encoding a structural or functional component  
being needed for the synthesis or maintenance of the cell  
20 envelope and ii) a gene encoding said desired product;  
transforming a suitable host bacterium having a defect in  
the chromosomal gene for said structural or functional component  
with said combined extra-chromosomal element;  
cultivating the transformed bacterium in a suitable nutrient  
25 medium; and  
recovering the desired product from the culture medium.

19. A method for the production of a desired product  
in transformed bacteria which method comprises:

cultivating in a suitable nutrient medium bacteria having a  
30 defect in a chromosomal gene needed for the synthesis or  
maintenance of the cell envelope, said bacteria containing a  
combined extra-chromosomal element being capable of suppressing  
the requirement caused by the chromosomal defect of the host  
bacterium, said combined extra-chromosomal element also  
35 containing a DNA-sequence encoding said desired product; and  
recovering the desired product from the culture medium.

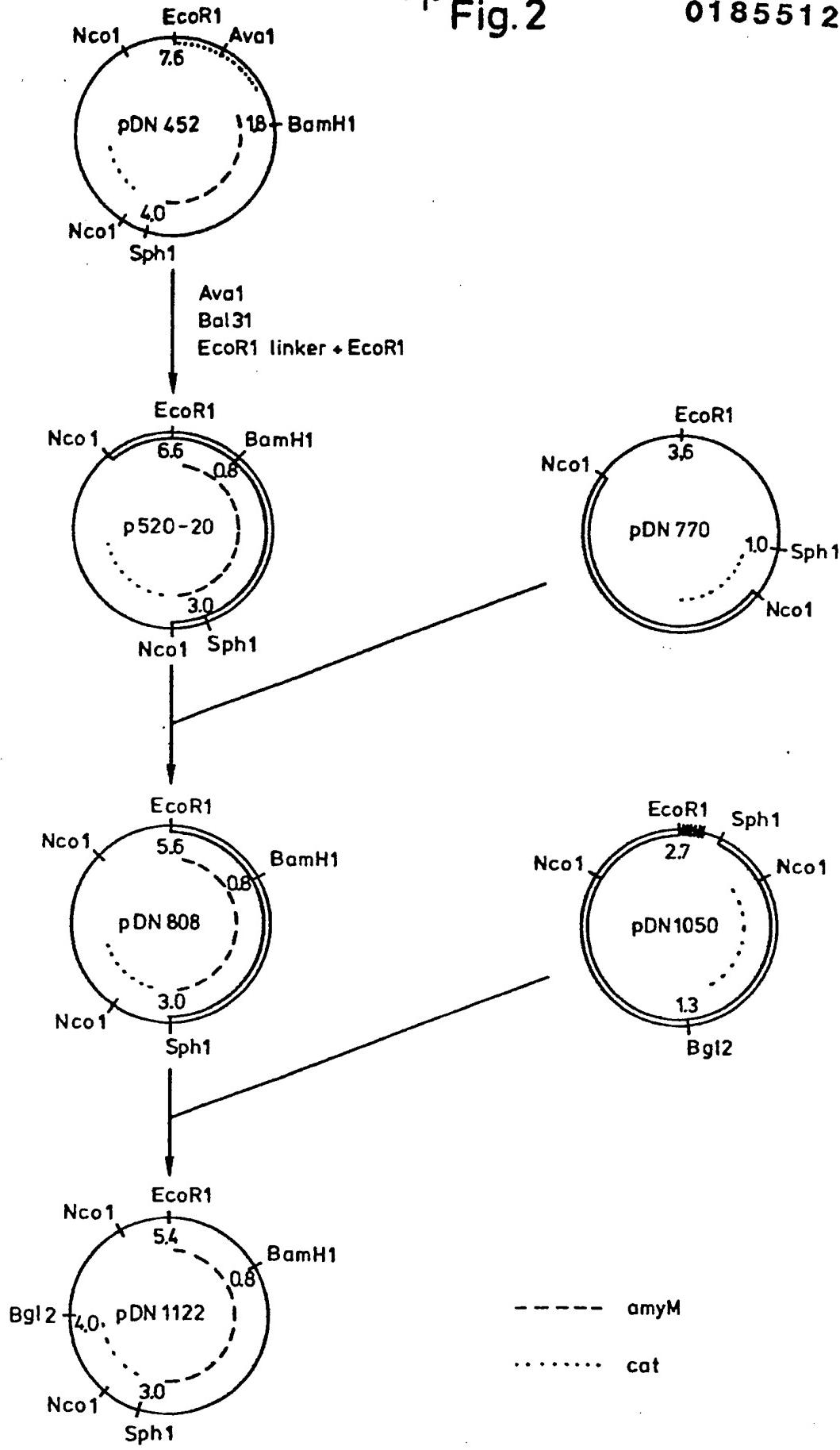
Fig.1

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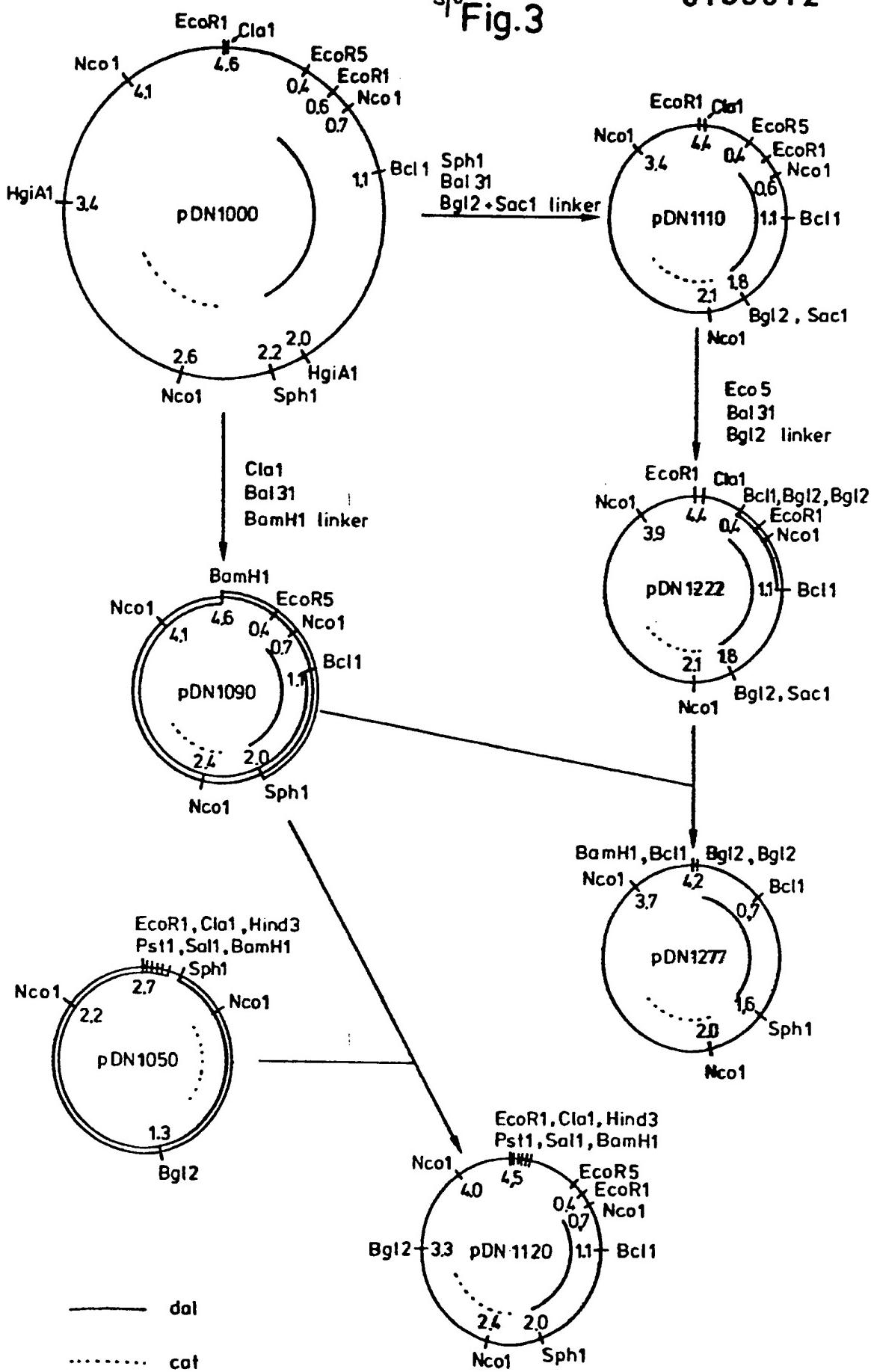
2/6 Fig. 2

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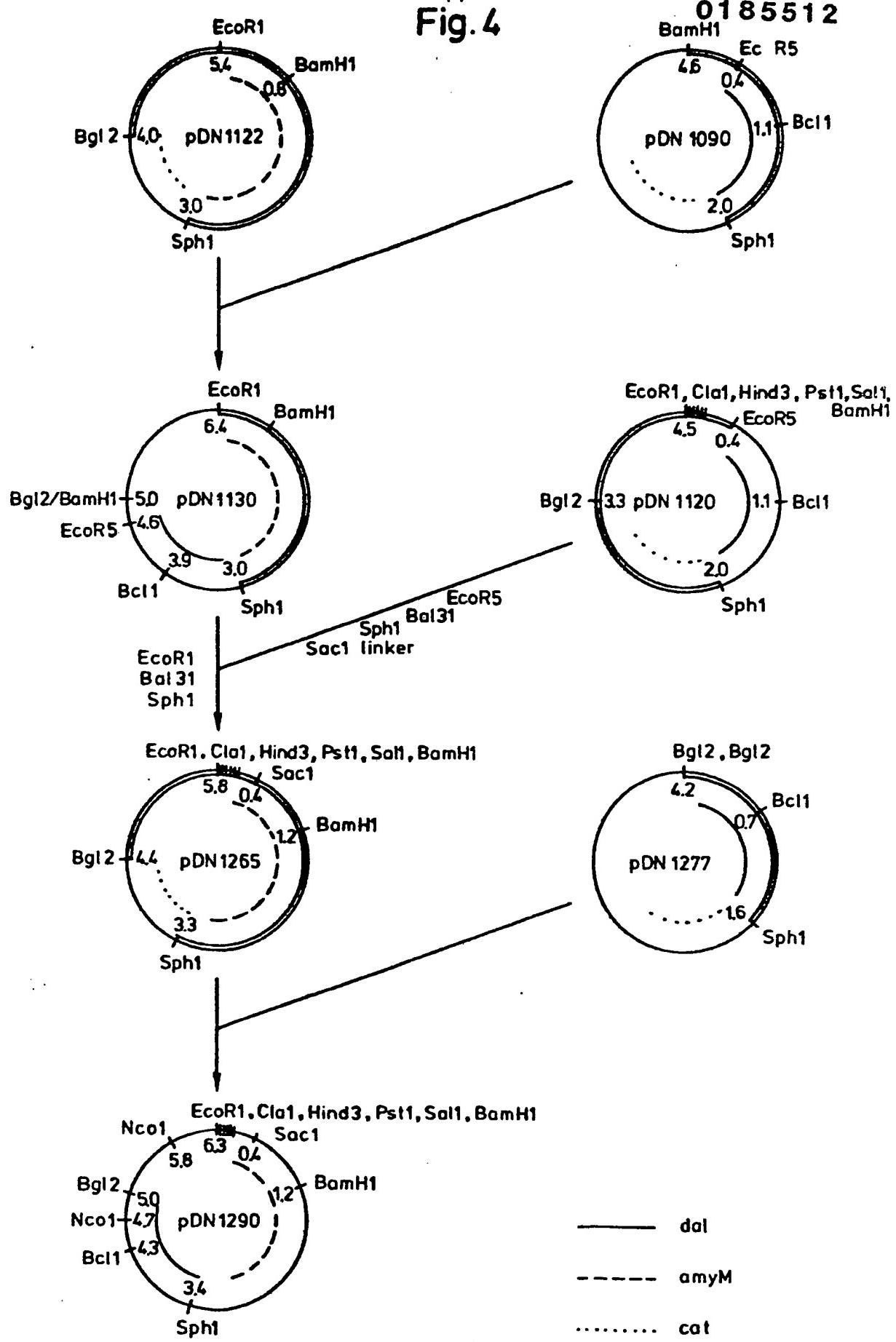


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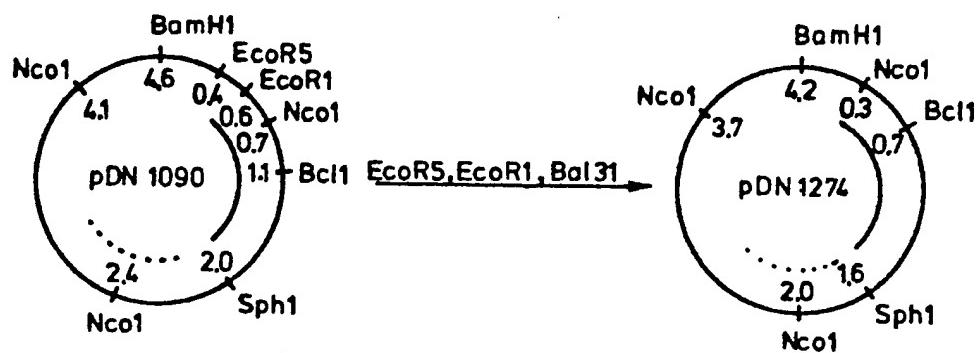


4/6  
Fig. 4



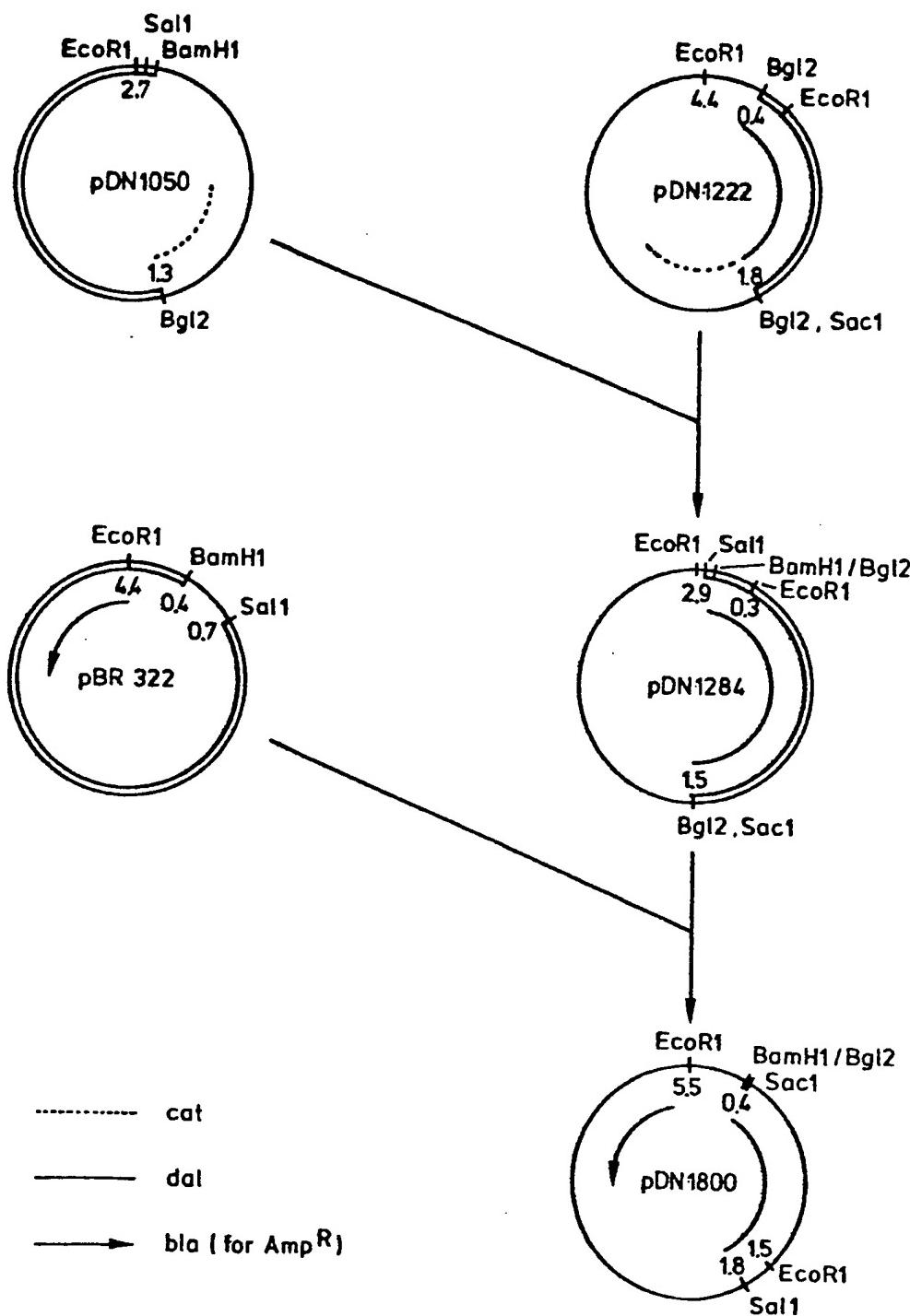
S/6  
Fig.5

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Fig.6

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DOCUMENTS CONSIDERED TO BE RELEVANT			EP 85309020.7
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
D,A	JOURNAL OF BACTERIOLOGY, vol. 115, September 1973 (Baltimore, USA)  M.J. DUL et al. "Genetic Mapping of a Mutant Defective in D,L-Alanine Racemase in Bacillus subtilis 168" pages 1212-1214  * Totality *  --	1-3,6-8	C 12 N 15/00 C 07 H 21/04 C 12 N 1/20 //C 12 R 1:07 C 12 R 1:125 C 12 R 1:19
A	EP - A2 - 0 120 629 (UNIVERSITY OF DELAWARE)  * Abstract *  --	1-5	
A	EP - A2 - 0 106 542 (A/S ALFRED BENZON)  * Abstract *  --	1	
A	EP - A1 - 0 012 494 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA)  * Abstract *  ----	1	C 12 N C 07 H
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl 4)
Place of search		Date of completion of the search	Examiner
VIENNA		12-03-1986	WOLF
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	



European Patent Office

Application number:

85309185512

**DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4,  
OF THE EUROPEAN PATENT CONVENTION**

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

**IDENTIFICATION OF THE MICRO-ORGANISMS**

Accession numbers of the deposits:      N C I B 12029 .  
    " 12030 .  
    " 12181 .